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13. ABSTRACT (Maximum 200

In the course of the past year, we have completed a meta-analysis of published studies, which confirms the expression and prognostic role of the MDR1 mechanism of resistance in human breast cancer. In our effort to obtain effective inhibitors of this mechanism, we have synthesized some novel progesterone analogs Among these, PgA4 is in vitro about 30-fold more potent a MDR1 inhibitor than the parental compound. Obtaining an MTD in vivo is limited by PgA4 low water solubility. We have defined a cyclodextrin-based formulation which, because of increased PgA4 solubility and decreased local tissue toxicity, will allow the in vivo administration of repeated and higher doses of PgA4. The sulfur in the PgA4 side chain is potentially susceptible to oxidation in the liver. Thus, we have begun synthesis of a PgA4 analog where a carbon atom replaces the sulfur in the side chain, making it less susceptible to a possible metabolic inactivation. We have established an assay to measure doxorubicin accumulation in xenografts, and detected doxorubicin in LCC6 human breast cancer xenografts. We will use the ratio of intra-tumor doxorubicin accumulation in MDR1-positive and -negative xenografts as a preliminary endpoint for the evaluation of our drugs' in vivo activity.

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<u>FOREWORD</u>

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INTRODUCTION

Most advanced breast cancers, which are exposed to and initially respond to cytotoxic chemotherapy, eventually develop a multidrug resistant (MDR) phenotype. This phenotype is characterized by cross-resistance to drugs to which the tumor has not been exposed, and is responsible for the failure of current systemic treatment regimens. The development of strategies specifically targeting the mechanisms responsible for multidrug resistance represents a logical approach to improve the efficacy of systemic therapy. The MDR phenotype in heavily pretreated breast cancer is likely to involve more than one mechanism. However, in the significant fraction of breast cancers that respond to first-line chemotherapy, the initial drug resistance may reflect only one, or a relatively few, resistance mechanisms. This subset of patients would appear to represent the ideal target of studies aimed to evaluate the prognostic role of specific mechanisms of resistance and the therapeutic efficacy of reversing agent strategies.

One of the best characterized mechanisms of multidrug resistance is mediated by the function of gp170, the product of the MDR1 gene. This membrane glycoprotein confers a MDR phenotype apparently by effluxing anticancer compounds belonging to several classes, including the anthracyclines, epipodophyllotoxins, Vinca alkaloids and taxanes. Substrates for gp170 also include many non-cytotoxic substrates, several of which have been investigated for their ability to reverse gp170-mediated resistance. Most appear to function as competitive inhibitors of gp170-mediated drug efflux. However, the majority of these reversing agents were originally designed for different therapeutic purposes, and the toxicities that limit their clinical usefulness is partly related to their original activity. Analogues of these drugs also have been used to increase the specificity of MDR1 effect while reducing toxicity, but none have clearly demonstrated consistent benefit in clinical trials.

We have adopted a rational analog design approach to generate agents specifically targeting the gp170 mechanism. Steroids are natural gp170 substrates, and progesterone exhibits the highest MDR1 reversing activity among the natural steroids, while also being relatively non-toxic. Consequently, we chose progesterone as our lead compound, and introduced chemical modifications based on the objectives of both increasing the MDR1 reversing while decreasing endocrine activity.

Bulky substitutions in C7 of the steroid nucleus were reported to decrease hormonal activity [1]. Moreover, length of the spacer in compounds comprising two linked structures has been shown to be critical for MDR1 reversing activity [2, 3]. We have designed and synthesized a first generation of analogs where the C7 position of progesterone is substituted with a bulky moiety. As expected, we observed both an increase of MDR1 reversing and a decrease of hormonal activity. One of the analogs, PgA4, is 35-fold more potent than the lead compound and up to 10 times more potent than verapamil as a MDR1 inhibitor. Evaluations carried out using promoter linked reporter constructs confirmed that PgA4 is essentially devoid of its progesterone agonist, and glucorticoid agonist/antagonist, activities. These data were reported in detail and illustrated in the original application.

The specific aims of this project are to: (1) evaluate PgA4's *in vivo* toxicity and MDR1 reversing activity; (2) optimize the pharmacophore on PgA4 by altering the bridge length and F ring on the C7 moiety; (3) determine the nature of the interaction between the C7-progesterone analogs and gp170. It was also important, at the beginning of our study, to ascertain the expression and prognostic impact of MDR1/gp170 expression in human breast cancer. Months 1 to 12 have been

dedicated to the completion of a meta-analysis study of studies evaluating MDR1 expression and prognostic role in human breast cancer (preliminary data were presented with the original research proposal), and to the pursuit of Specific Aim 1. While the testing of PgA4 activity *in vivo* is now our primary objective, we have begun work on synthetic pathways to an analog potentially less susceptible to oxidation of the thiol linker. This oxidation could occur in the liver, and might adversely affect the pharmacokinetics pf PgA4.

BODY

Experimental Methods, Assumptions and Procedures

Assumptions and Approach. We have chosen to alter the order of some of the tasks, to more effectively complete the project. We believe that this flexibility is important to the expeditious and successful completion of the overall goals of the study. We chose first to complete the meta analysis, presented as preliminary data in the original application, since this has become a controversial area and is central to the rationale for the funded studies. We have completed this analysis, the results of which were recently published in *J Natl Cancer Inst*, [4] and were the subject of two Editorials in the same issue [5,6]. We are currently responding to one of the Editorials, to correct several erroneous comments in the Editorial by Kaye [5].

We also have completed submission of a patent application on the compounds, which were previously covered by a Preliminary (one year) patent. Thus, we now have extended full patent protection for our compounds and the proposed modifications indicated in the original application. This also allows us to prepare several publications. We will complete the final chemical analyses of some of the less active compounds in the initial series, so that we can publish these together. We would hope to have these finished within the next weeks, and the first synthesis/activity manuscript submitted shortly thereafter.

As a result of ongoing interactions within the research group, we discussed the possibility of oxidation of the thiol linker, which could occur in the liver and might adversely affect the pharmacokinetics of PgA4. While a major component of Aim 1 was to continue the preliminary *in vivo* studies, we felt that it might be better to first synthesize a potentially more metabolically stable analogue. We found several citations to synthetic approaches that would allow us to generate a carbon-linked pharmacophore, which should be resistant to the potential oxidation that might occur with the thiol-linked pharmacophore. As is apparent below, we have begun work on synthetic pathways for the carbon-linked compounds, in preference to more detailed *in vivo* studies with the thiol-linked PgA4. If we are unsuccessful in these approaches, which seems unlikely in the long run since others have generated carbon linked analogues of steroids as inhibitors of aromatase, we would return to PgA4.

A further prerequisite for the *in vivo* studies is the identification of an appropriate vehicle, since the compounds are, of necessity, relatively hydrophobic. We tried several formulations, and now present data with our current approach, using cyclodextrins. To be able to complete the *in vivo* studies, it also is necessary to have a nonradioactive assay where we can directly measure intratumor drug concentrations. Our intention is to initially compare intratumor levels in our transfectants and

controls, using a study design where transfectants are inoculated on one flank and control cells on the other. This approach should control for interanimal variability, and allow for a more direct demonstration of the ability of our compounds to increase drug accumulation *in vivo*. We have now established such an assay and successfully detected Doxorubicin in MCF7/LCC1 (wild type) cells.

Meta-analysis studies. A meta-analysis was carried out on 31 published studies evaluating the MDR1/P-glycoprotein expression in human breast cancer [4]. The proportion of tumors expressing MDR1/P-glycoprotein was calculated for each study and, where appropriate, in specific subgroups [7]. Chi-square tests of homogeneity [8] weighted means and weighted linear regression [9] were used to evaluate the heterogeneity of proportions across studies. Maentel-Haenszel methods [10] were used to calculate the pooled relative risks (RRs) for the association between prior chemotherapy and the induction of MDR1/P-glycoprotein expression and between MDR1/P-glycoprotein expression and clinical outcomes. Heterogeneity of RRs across studies was calculated by chi-squared tests [10].

Formulation studies. We compared the solubility of PgA in formulation A (used in initial evaluations and described above) and B (20% 2-hydroxypropyl-β-cyclodextrin in PBS) [11, 12]. Based on the evaluation of the UV and visible spectrometric profile of PgA4, we selected 245 nm as the ideal wavelength for all subsequent evaluations of PgA4 concentration. In order to evaluate PgA4 solubility, we used the method described by Pitha and Pitha [12]. Briefly, saturated solutions of PgA4 were obtained by stirring an excess amounts of PgA4 at room temperature overnight in each vehicle. Samples were centrifuged at 13,000 rpm and supernatants filtered through Amicon micropure separators. The 245 nm absorbance of samples obtained as 1/10, 1/100, and 1/1000 dilutions of each PgA4 saturated formulation was evaluated, and PgA4 concentrations were calculated by interpolation on the respective standard curve (obtained by serial dilution of PgA4 100 μM in the respective vehicle).

Intratumor Doxorubicin. LCC6 cells (1×10^6) were inoculated *s.c.* into the mammary fat pads of 5 NCR *nu/nu* athymic nude mice and solid tumors grown to an average volume of 130 mm³. Mice were then treated with doxorubicin 8 mg/Kg *i.v.* 24 hours later, the mice were sacrificed and the LCC6 tumors recovered for doxorubicin analysis. Tumor doxorubicin content was evaluated according to the method described by Broggini et al.[13]. Tumor samples were homogenized in water and deproteinized with 33% silver nitrate. Samples were extracted with isopropanol and the organic phase collected and evaporated to dryness under vacuum. The dried samples so obtained were redissolved in the HPLC mobile phase using daunomycin as an internal standard. Samples were run in the HPLC and the doxorubicin peak fluorescence read at an excitation wavelength of 475 nm and emission of 580 nm.

Design and synthesis of a PgA4 analog with carbon-sulfur bond (C7 moiety) replaced by carbon-carbon bond

The definition of the synthetic pathways for the synthesis of this analog of PgA4 is the goal of our present effort. Structures and synthetic pathways are, consequently, described in the following Results section.

RESULTS AND DISCUSSION

Meta-analysis studies

The results from these studies are most effectively seen in the attached reprint [4]. Briefly, the average proportion of breast cancers expressing MDR1/P-glycoprotein was 41.2%. However, overall heterogeneity across studies was highly significant and was largely to be ascribed to a switch, over time, from RNA-hybridization assays to immunohistochemistry. The proportion was significantly higher in patients previously treated with systemic therapy (RR=1.77). Patients whose tumors expressed MDR1/P-glycoprotein were significantly more likely to fail to respond to systemic chemotherapy: overall RR was 3.21, but increased to 4.19 in patients whose tumors were positive after chemotherapy. There was no significant heterogeneity among these studies. MDR1/P-glycoprotein appeared to be a prognostic independent of others like tumor size, grade, histology and estrogen receptor status and lymph node metastases.

Though a causal role of MDR1 in affecting breast cancer response to chemotherapy remains to be proven, the results of this meta-analysis are consistent with such role, showing a correlation between MDR1 expression and response to chemotherapy. These results illustrate the clinical relevance of this project, and the validity of MDR1-reversing strategies for the management of breast cancer. This study was presented at the recent DOD Era of Hope meting in Washington D.C. as a poster and podium presentaton by Dr. Clarke.

Formulation studies

Our early observations prompted us to try and improve PgA4 solubility, to increase the dose of PgA4 that can be administered *in vivo*. While we have already planned chemical modifications to make PgA4 more soluble, we have decided to first try to optimize solubility through formulation. An ideal drug vehicle should both increase drug solubility and exhibit low local and systemic toxicity. The solubility of some steroids, including progesterone and several progesterone derivatives, is increased by the use of cyclodextrins [11; 12]. The cyclodextrins are cyclic oligosaccharides, *e.g.,* 2-hydroxypropyl-β-cyclodextrin, which are highly water soluble and can increase steroid solubility through the formation of inclusion complexes. The steroids bind to the cyclodextrin hydrophobic cavity. 2-hydroxypropyl-β-cyclodextrin also has very low toxicity, even when administered parenterally [14]. The solubility of PgA4 in the 2-hydroxypropyl-β-cyclodextrin formulation was 1.0 mM, i.e., about 5-fold higher that in the early formulation (0.2% chloroform, 9.8% ethanol, 40% propylene glycol). We also confirmed that the new vehicle exhibits no acute local or systemic toxicity. This improvement in vehicle will likely also be relevant to the new carbon-linked compounds, which would be expected to exhibit essentially similar solubility to PgA4.

We now can perform more detailed toxicity studies, using concentrations up to 5-times higher than on our initial studies. Once we have assessed toxicity at the maximum tolerated dose, we will be able to initiate the *in vivo* reversing studies described in the original application. MDR1 inhibitors may cause more than additive toxicity when combined with anticancer drugs, due to pharmacokinetic interactions mediated by inhibition of the P-glycoprotein expressed in normal

tissues with an excretory or barrier function, *e.g.*, liver, kidney or the capillary endothelial cells of the blood-brain barrier [15, 16]. Consequently, preliminary MTD/LD10 evaluations, designed to optimize the toxicity of drug combinations, may be required for drugs that may eventually turn out to be completely inactive *in vivo*.

Accumulation of adriamycin in human breast cancer cell xenografts
Consistent with the known mechanism of action of P-glycoprotein, MDR1 expressing tumors have been shown to accumulate lower intratumor concentrations of MDR1 substrates. Evidence of differential doxorubicin accumulation in MDR1-positive and -negative tumors is apparent a few hours after doxorubicin administration [17, 18]. We decided to test whether we could detect doxorubicin in tumors, and whether this might provide a useful early endpoint for the evaluation of *in vivo* MDR1-reversing activity. This now seems likely, since we could detect doxorubicin in the wild type MDA435/LCC6 tumors. Doxorubicin concentration in LCC6 tumors was 48.2 ± 9.8 ng doxorubicin/mg protein (mean and standard error).

In the next year, we will compare doxorubicin accumulation in LCC6 and LCC6/MDR1 both *in vitro* and *in vivo* in the presence and absence of PgA4 and/or a carbon-linked pharmacophore analogue (see next section).

Design and synthesis of a PgA4 analog where the thiol linkage at C7 is replaced by a carbon link
We considered the possibility that the C7 linkage in PgA4 might be sensitive to oxidation. Thus, we have designed and begun synthesis of such analogues where the carbon-sulfur bond in the C7 moiety bridge is replaced by a carbon-carbon bond.

We have constructed the following synthetic steps (fig.1): (1) Oxidation of progesterone (compound 1 of fig. 1) to obtain 6-dehydroprogesterone (compound 2); (2) protection of the ketone in position 23 (compound 3) ; (3) conjugate addition of 4'-amino-benzylbromide (compound 4); (4) reaction with α-phenethyl-isocyanate. Steps 1 and 4 are already used in the synthesis of PgA4 and are described in the Materials and Methods section of the original research proposal.

We are currently working on the method to protect the ketone at position C23 (step 2) prior to pharmacophore addition at C7 (step 3). Step 2 appears necessary, as early attempts to directly conjugate unprotected 6-dehydroprogesterone were unsuccessful. However, attempts at directly protecting 6-dehydroprogesterone (from compound 2 to 3 in fig. 1) also failed. Efforts were then focused on an alternative route (fig. 2) that involves protection of a single ketone group (at C3) followed by oxidation to the desirable α-β, δ-γ unsaturated ketone (compound 6 in fig.2). This was accomplished successfully and compound 6 was then treated with a methanolic solution of potassium hydroxide to obtain the desired free alcohol (compound 7). Synthesis of compound 3 from compound 7 was attempted by two different approaches, so far with very low yields. Only partial oxidation was obtained, and attempts to purify the product using silica gel flash chromatography appeared to deprotect much of the protecting group. Attempts to alkylate compound 3 have been so far unsuccessful. Other neutral methods are currently being examined to avoid deprotection of the ketal back to the starting ketone during purification.

These studies were initially performed by a graduate student in our collaborator's laboratory.

This proved less than satisfactory, due largely to inexperience, and we now plan to hire a more experienced postdoctoral fellow to repeat these studies.

Recommendations in Relation to the Statement of Work

STATEMENT OF WORK

OBJECTIVE 1: *IN VIVO* EVALUATION OF PGA4'S TOXICITY AND POTENCY FOR gp170-REVERSAL
Task 1:   Estimation of MTD/LD$_{10}$: Months 1-8.
Task 2:   Estimation of potency: Months 6-12.

OBJECTIVE 2: BRIDGE OPTIMIZATION AND *IN VITRO* POTENCY
Task 3:   Synthesis of PgA4 analogues to optimize the pharmacophore's bridge length: Months 12-18.
Task 4:   Determination of *in vitro* potency of bridge-optimized analogues: Months 12-24.

OBJECTIVE 3: F-RING OPTIMIZATION AND *IN VITRO* POTENCY
Task 5:   Synthesis of analogues of bridge-optimized PgA4 analogue to optimize the pharmacophore's potency: Months 24-30.
Task 6:   Determination of *in vitro* potency of bridge-optimized analogues: Months 24-36.

OBJECTIVE 4: *IN VIVO* EVALUATION OF BRIDGE/F-RING OPTIMIZED ANALOGUES
Task 7:   Estimation of MTD/LD$_{10}$: Months 36-42.
Task 8:   Estimation of potency: Months 36-48.

OBJECTIVE 5: MECHANISTIC STUDIES
Task 9:   gp170 binding, VBL accumulation, influx and efflux, effects og gp170 expression: Months 30-48.

We have postponed carrying out tasks 1 and 2 due to the poor solubility of PgA4 in the initial formulation. This formulation has now been defined.

We also have established a more adequate endpoint for the preliminary evaluation of the *in vivo* MDR1-reversing activity (intratumor doxorubicin evaluation) of all analogues. By not being affected by delayed toxicity, this model will allow a preliminary detection of MDR1 reversing activity prior to MTD evaluations. It also will prevent costly and lengthy MTD evaluations in agents devoid of any MDR1 reversing activity. Agents active by this preliminary endpoint will undergo evaluation of acute/subacute toxicity and of MDR1 reversing potency, using tumor growth delay and/or survival endpoints.

We have also anticipated the need for a PgA4 analogue less sensitive to metabolic inactivation and started studying the relative synthetic pathways.

Having defined PgA4 formulation and an adequate *in vivo* preliminary endpoint, we are now set to start the evaluation of the *in vivo* MDR1 reversing activity. In the course of the second year of this project we will also evaluate the *in vitro* and *in vivo* activity of PgA15 ("carbon-bridged" analogue of PgA4) and compare it with that of PgA4. We expect the *in vitro* and *in vivo* activity to be respectively unaltered and improved. If this expectation is met, we may consider to modify similarly the other planned progesterone analogs.

No further changes are recommended to the original plan at this point, and Tasks 3 and 4 (bridge optimization and *in vitro* potency) stand as defined in the Statement of Work for the second year of this Project.

CONCLUSIONS

The data we have obtained support the relevance of the MDR1 mechanism of drug resistance in human breast cancer. Gp170 is expressed in a significant proportion of breast cancers; this proportion is increased following systemic therapy, and correlates with a lower response rate to chemotherapy.

PgA4, the most potent of our first generation C7 progesterone analogues, can now be formulated at concentrations about 5 fold higher than those originally achievable (up to about 1 mg/ml). This new formulation is based on the use of 20% 2-hydroxypropyl-β-cyclodextrin. The new vehicle has the additional advantage of being less toxic locally and systemically and this will further increase the dose that can be delivered *in vivo* by allowing repeated *s.c.* administration of larger volumes of the drug preparation.

We have defined a model based on intratumor accumulation of doxorubicin as an early *in vivo* endpoint to assess the activity of PgA4 and its subsequent analogues. We will now proceed with the evaluation of PgA4 *in vivo* MDR1 reversing activity.

We also will proceed with the goals outlined for months 13 to 24, by synthesizing and testing a second generation of PgA4-derived analogs aimed to optimized the C7 moiety bridge length. Where possible, these will be based on the carbon-carbon rather than carbon-thiol linkage, provided that our present work to define the synthetic pathways to the carbon-carbon lead are successful and we can demonstrate that this new analogue has retained antigp170 activity. The activity of the carbon-carbon analogue will be established as described in the original application for assessing PgA4 activity. PgA4 will be included for direct comparison in all of these studies.

In conclusion, we believe that this project is progressing essentially as planned. We do not foresee any major problems in completing the studies as described, since we have established the necessary methodology for measuring intratumor drug, and have increased our ability to deliver drug 10-fold or more. Thus, we should be able to determine *in vivo* toxicity and obtain preliminary estimates of *in vivo* potency within the next 12 months. We also should be able to complete synthesis of several of the proposed modifications within this time.

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FIGURE LEGENDS

Fig. 1 Proposed synthetic steps to PgA15 (compound : (1) Oxidation of progesterone (compound 1) to obtain 6-dehydroprogesterone (compound 2); (2) protection of the ketone in position 23 (compound 3) ; (3) conjugate addition of 4'-amino-benzylbromide (compound 4); (4) reaction with alfa-phenethyl-isocyanate to obtain compound 5 (PgA15).

Fig. 2 Proposed alternative route to the protection of the ketone group in C23. Protection of the ketone group at C23 is achieved by first protecting the ketone group (at C3) followed by oxidation to the desirable α-β, δ-γ unsaturated ketone (compound 6). Compound 6 is then treated with a methanolic solution of potassium hydroxide to obtain the desired free alcohol (compound 7). Finally, compound 7 is oxidized to compound 3
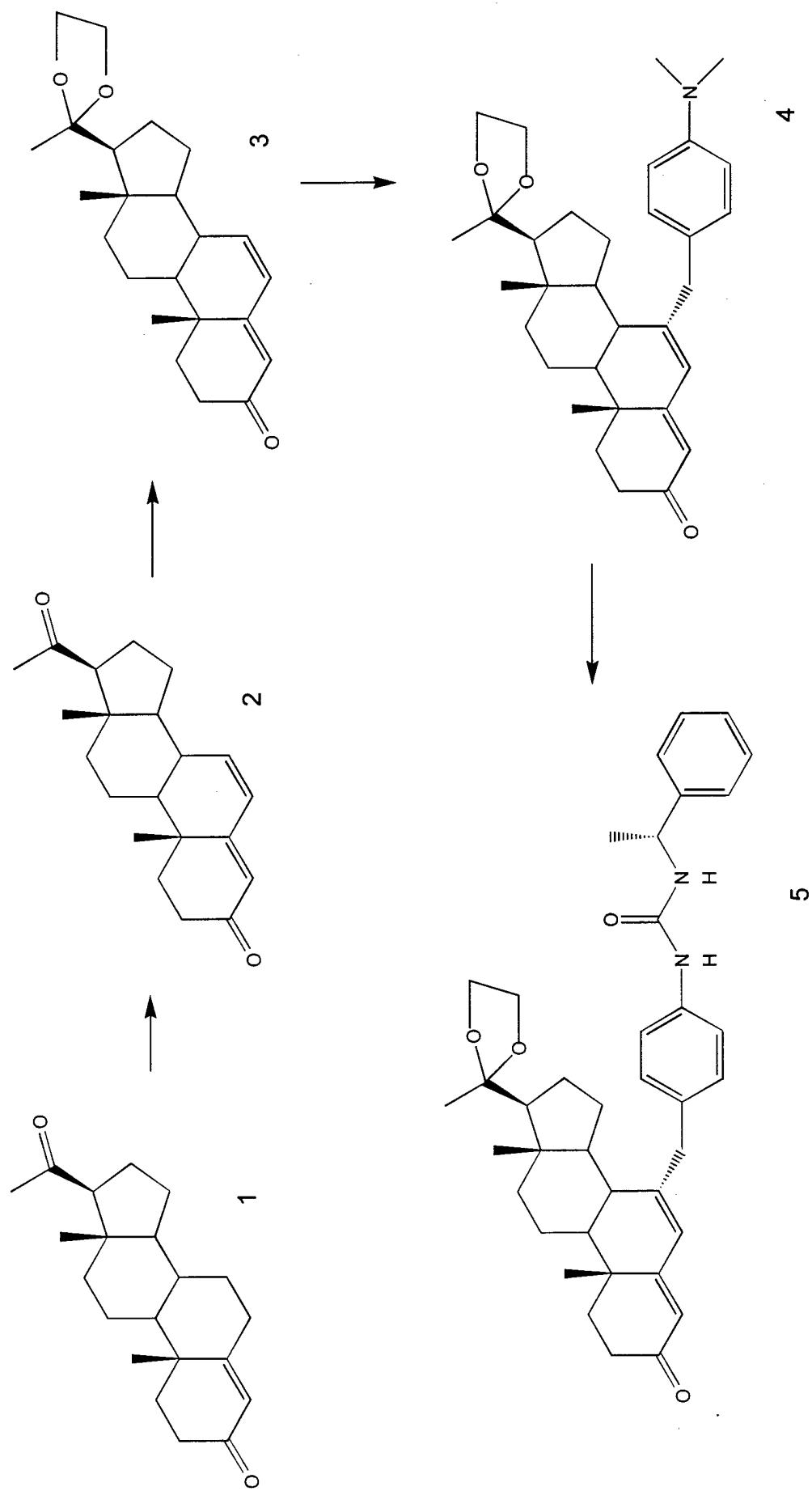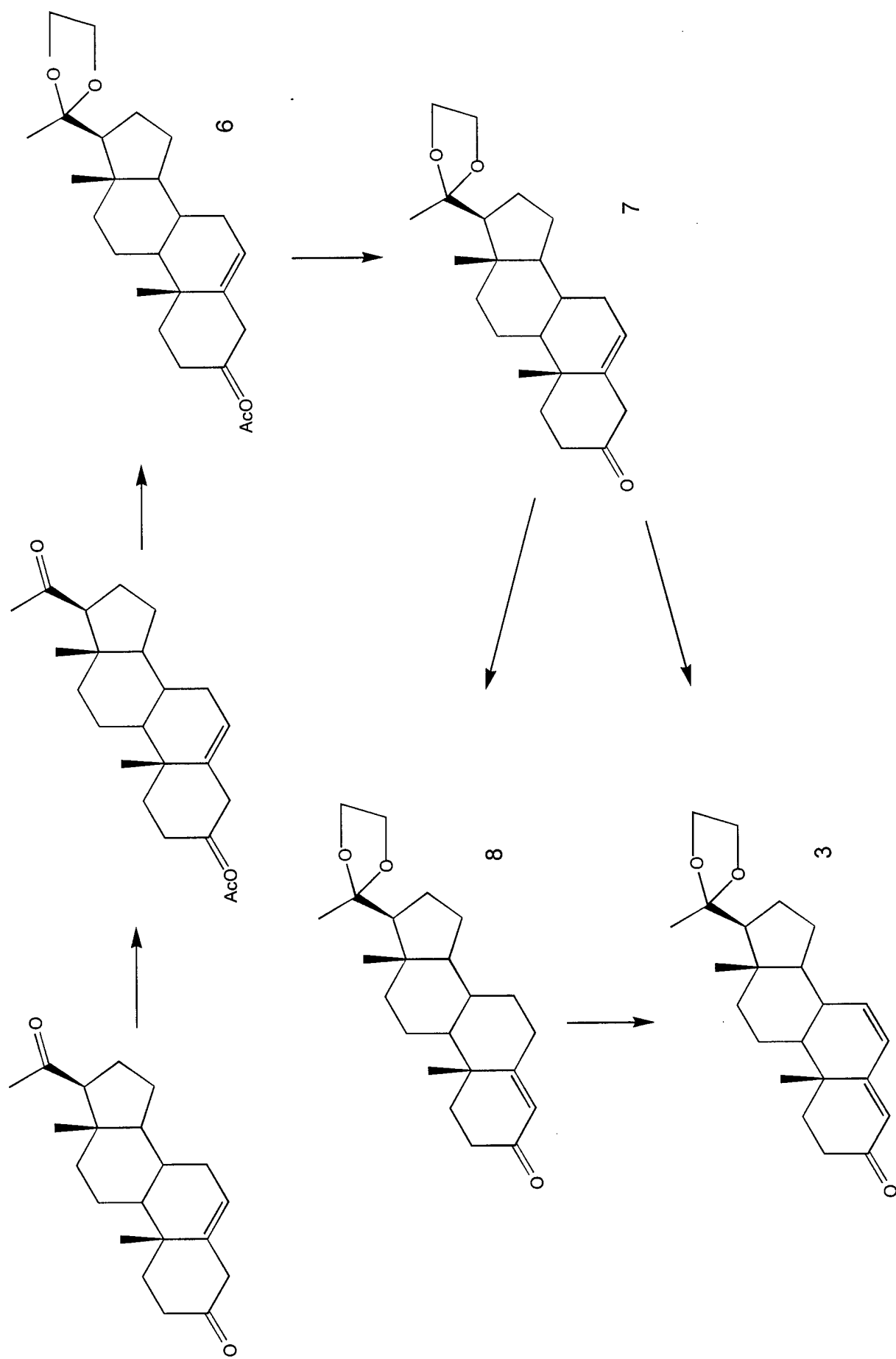
Fig. 1

Fig. 2